VERSION WITH MARKINGS TO SHOW CHANGES MADE IN THE SPECIFICATION

1. The changes relative to the previous version, in page 6, line 11, of the entire paragraph after "in the step (f)." are marked up as follows:

[Advantageously, the reactions of step (b) and steps (d) to (f) can be continuously completed under the same buffering condition (20mM Tris-HCl, pH8.3 at 25°C, 120mM KCl, 60mM $(NH_4)_2SO_4$, 8mM $MgCl_2$, 1M betaine, 7mM DTE and 2mM spermidine) during the cycling amplification procedure of the present invention. To overcome the nonspecific amplification of PCR in previous mRNA preparations, we designed high G-C content promoter-primers (SEQ ID. 3 and 5) annealed to the most 5'-end of a cDNA template in the sense orientation for generating full-length mRNAs using in-vitro transcription reactions (not PCR-based reactions) and therefore without the drawbacks of PCR. Because the capability of using transcriptional amplification in either the sense (mRNA) or both orientations, our invention advantageously provides more flexibility in the generation of either pure full-length mRNAs (FIG.1) or double-stranded RNA (mRNA/aRNA) mixtures (FIG.2), ready for a variety of biochemical applications such as full-length mRNA/cDNA preparation, probe preparation, in-vitro translation and gene knockout analysis (RNA interference).]

2. The changes relative to the previous version, in page 6, line 12 to page 7, line 2, of the last paragraph are marked up as follows:

The mRNAs can be prepared from a plurality of fixed cells, wherein said fixed cells are protected from RNA degradation and also subjected to permeabilisation for enzyme penetration. Those fixed cells include fixative-treated cultural cells, frozen fresh tissues, fixative-treated fresh tissues or paraffin-embedded tissues on slides. To increase the transcriptional production of mRNAs in the step (e), the promoter sequences are preferably incorporated into the 5'-ends of said second-strand cDNAs, including the primers of SEQ ID. 1, 3 and 5 whose annealing temperature is about 52~55°C for about 3~10 min in a consistently buffered condition (20mM Tris-HCL, pH 8.3 at 25°C, 120mM KCl, 60mM $(NH_4)_2SO_4$, 8mM $MgCl_2$, 1M betaine, 7mM DTE and 2mM spermidine) as

described in Examples 1, 4 and 5 respectively. In another aspect of this embodiment, said amplified mRNAs are preferably capped by P^1-5'-(7-methyl)-guanosine-P^3-5'-adenosine-triphosphate or P^1-5'-(7-methyl)-guanosine-P^3-5'-guanosine-triphosphate in the step (e) for further in vitro translation. On the other hand, the deoxynucleotide used in the tailing reaction of said first-strand complementary DNAs is either deoxyguanylate (dG) or deoxycytidylate (dC), and the average number of tailed nucleotides is larger than seven; most preferably, the number is about twelve. Advantageously, the final amplified mRNAs can be continuously reverse-transcribed into double-stranded cDNA by Tth-like DNA polymerase activity. The final double-stranded cDNAs are preferably cloned into competent vectors for further applications, such as transfection assay, differential screening, functional detection and so on.

3. The changes relative to the previous version, in page 8, line 6 to line 20, of the second paragraph are marked up as follows

The present invention is directed to a novel polymerase chain reaction method for mRNA amplification from single cells, named "RNA-polymerase chain reaction (RNA-PCR)". This method is primarily designed for differential screening of tissue-specific gene expressions in cell level, cloning full-length sequences of unknown gene transcripts, generating pure probes for hybridization assays, synthesizing peptides in vitro, and preparing complete cDNA libraries for gene chip technology. The purpose of the RNA-PCR relies on the repeating steps of reverse transcription, denaturation, double-stranded cDNA synthesis and in vitro transcription to bring up the population of mRNAs to two thousand folds in one cycle of above procedure. In brief, the preferred version (FIG.1) of the present invention is based on: 1) prevention of mRNA degradation (Example 1), 2) first reverse transcription and terminal transferase reaction to incorporate 3'-polynucleotide tails to the first-strand cDNAs (Example 2 or 5), 3) denaturation and then double-stranded cDNA formation based on the extension of specific promoter-primers complementary to the 3'-polynucleotide tails (Example 3 or 5), 4) transcription from promoter to amplify mRNAs up to two thousand folds per round (Example 3 or 5), and 5) repeating aforementioned steps to achieve desired RNA amplification (Example 5).

4. The changes relative to the previous version of the paragraph between page 8, line 21 and page 9, line 12 are marked up as follows:

Alternatively, the second preferred version (FIG.2) of the present invention is based on: 1) prevention of mRNA degradation (Example 1), 2) first reverse transcription to incorporate first promoters to the 5'-ends of first-strand cDNAs and then addition of polynucleotide sequences to the 3'-ends of the first-strand cDNAs (Example 2), 3) double-stranded cDNA synthesis based on the extension of second promoter sequences complementary to the 3'-polynucleotide regions of the first-strand cDNAs (Example 3), 4) transcription to amplify either aRNAs or mRNAs up to two thousand folds in the first round of amplification cycle (Example 3), and 5) repeating aforementioned cycling steps to achieve desired amount of RNAs (Example 4). As shown in FIG.2, the first promoter used here is different from the second promoter, resulting the control of transcription by adding different RNA polymerases. The first promoter is incorporated for aRNA amplification, whereas the second promoter is designed for mRNA amplification. By this way in conjunction with a reverse transcription step, we can choose to amplify aRNAs, first-strand cDNAs, mRNAs or second-strand cDNAs of interest, depending on which RNA polymerase and nuclease we use. Although the second and third preferred embodiments (FIGS.2 and 3) are more complicated than the first preferred embodiment (FIG.1), the principle and broad features of the second and third preferred embodiments are completely within the scope of the first preferred embodiment of the present invention.

5. The changes relative to the previous version, page 9, lines 13 to 27, of the last paragraph are marked up as follows:

As used herein, the first-strand complementary DNA (cDNA) refers to a DNA sequence which is complementary to a natural messenger RNA sequence in an A-T and C-G composition. The antisense RNA (aRNA) refers to an RNA sequence which is complementary to a natural messenger RNA sequence in an A-U and C-G composition. And, the oligo(dT)-promoter sequence refers to an RNA polymerase promoter sequence coupled with a poly-deoxythymidylate (dT) sequence in its 3'-end, of which the minimal

number of linked dT is seven; most preferably, the number is about twenty-six. The sense sequence refers to a nucleotide sequence which is in the same sequence order and composition as its homolog mRNA, whereas the antisense sequence refers to a nucleotide sequence which is complementary to its respective mRNA homologue. On the other hand, the oligo(antisense polynucleotide)-promoter sequence refers to an oligonucleotide sequence which is complementary to the polynucleotide-tail of said polynucleotide-tailed cDNAs and also linked to an RNA polymerase promoter in its 5'-end. And, the Tth-like DNA polymerases refer to RNA- and DNA-dependent DNA polymerases with reverse transcription activity, such as AMV, M-MuLV, HIV-1 reverse transcriptases and C. therm. Polymerase.

6. The changes relative to the previous version, page 10, lines 1 to 16, of the first paragraph are marked up as follows:

[By improving the methods of in-vitro transcription and in-cell RT-PCR (Embleton et.al., *Nucleic Acid Res.* (1992)), we] We invent [the] a thermal cycling amplification [of] procedure for reproducing intracellular full-length mRNAs (Lin et. al. *Nucleic Acid Res.* 27: 4585-4589 (1999)). This cycling procedure preferably starts from reverse transcription of intracellular mRNAs with Tth-like DNA polymerases (such as reverse transcription activity of C. therm. Polymerase which is initiated with primers (SEQ ID.1, 3 or 4) at about 65~72°C for about 30~60 min as described in Examples 2, 4 and 5 respectively), following a tailing reaction with terminal transferases (at about 37°C for about 15~20 min as described in Examples 2, 4 and 5) and then denaturation of resulting mRNA-cDNA hybrid duplexes (at about 94°C for about 2~3 min as described in Examples 2, 4 and 5). After renaturation (at room temperature for 1 min or at about 52°C for about 3 min) of above tailed cDNAs to specific promoter-linked primers (SEQ ID.3 or 5), double-stranded cDNAs are formed by Tth-like DNA polymerase, such as C. therm. DNA polymerase activity at about 70°C for about 5 min (Examples 3 and 4) or Taq DNA polymerase activity at about 72°C for about 7 min (Example 5). And then, promoter-specific RNA polymerase(s), T7 or SP6 RNA polymerase in Example 3, 4 or 5 respectively, is added to accomplish the transcriptional amplification of intracellular

mRNAs at about 37°C for about 1~3 hours. The novelties of this amplification cycling procedure of the present invention are as follows: 1) single copy rare mRNAs can be increased up to 2000 folds in one round of amplification without mis-reading mistakes, 2) the mRNA amplification is linear and does not result in preferential amplification of abundant mRNA species, 3) the mRNA degradation is inhibited by fixation, and 4) the final mRNA products are of full-length and can be directly used to generate a complete cDNA library or synthesize proteins in vitro (Shi-Lung Lin et.al. *Nucleic Acid Res.* (1999)).

7. The changes relative to the previous version in page 15 of the entire paragraph after line 20 are marked up as follows:

[The Example 1 describes a preferred step for the prevention of intracellular RNA degradation before the step (a) of the present invention. The Examples 2-4 are directed to each step of the second preferred embodiment (FIGS.2 and 3), while the Example 5 is draw to the first preferred embodiment (FIG.1) published in *Nucleic Acid Res.* 27: 4585-4589 (1999). Since previous methods failed to preserve the complete 5'-end of an RNA sequence for amplification due to the lack or loss of specific primer binding sites or due to the failure of the PCR reaction through polyG-C tails, our invention overcomes this bottleneck by designing special promoter-primers annealing to the 5'-added tail of a reverse-transcribed cDNA template in the sense orientation for generating full-length mRNAs using in-vitro transcription reactions (not PCR-based reactions). Although some commercialized buffering conditions for each enzymatic step component (Examples 2-4) are slightly different, we have designed a new RT&T buffer (as shown in Example 5) to unify the optimized condition for continuously thermocycling amplification of mRNAs. The changes of reacting temperatures during the thermocycling amplification is also specially designed (as shown in Examples 1-5) to fulfill the maximal activities of each enzymatic step, depending on the property of the enzyme involved. It has been proven that such unified conditions are capable of amplifying full-length mRNAs up to 5kb with reproducible results from as few as twenty cells (Lin et.al, 1999). The present invention is useful when linear amplification is required or only picogram starting materials can be

acquired for mRNA/cDNA amplification. Its results not only provide molecular diagnosis of cancerous genes in vivo but also increase the resolution of current genetic research to the single-cell scale.].

VERSION WITH MARKINGS TO SHOW CHANGES MADE IN THE CLAIMS

The changes relative to the previous version of the rewritten claim(s) 10-14, 16-18, 20, 30-31, 33, and 35 are marked up as follows.

In claim 10 (amended). The method as defined in Claim 9, wherein said first primer sequences [are coupled to an RNA polymerase promoter and] contain about eight to about thirty copies of deoxythymidylates.

In claim 11 (amended). The method as defined in Claim 1, wherein said denatured polynucleotide-tailed first-strand complementary DNAs are formed at temperature ranged from [about 90°C to about 100°C] about 94°C.

In claim 12 (amended). The method as defined in Claim 1, wherein said DNA polymerase activity is an enzyme activity selected from the group consisting of [E. coli DNA polymerase 1, Klenow fragment of E. coli DNA polymerase 1, T4 DNA polymerase, Taq DNA polymerase, Pwo DNA polymerase, Pfu DNA polymerase and Tth-like DNA polymerases, C. therm. Polymerase] Taq DNA polymerases and Tth-like DNA polymerases.

In claim 13 (amended). The method as defined in Claim 12, wherein said DNA polymerase activity is achieved by [Tth-like DNA polymerase with reverse transcriptase activity] C. therm. polymerases.

In claim 14 (amended). The method as defined in Claim 12, wherein said DNA polymerase activity is performed at temperature [ranged from about 35°C to about 85°C] about 70°C.

In claim 16 (amended). The method as defined in Claim 15, wherein said RNA polymerase promoter is selected from the group consisting of [T3, T7, SP6 and M13] T7 and SP6 RNA polymerase promoter.

In claim 17 (amended). The method as defined in Claim 1, wherein said transcription is an RNA polymerase activity selected from the group consisting of [T3, T7, SP6 and M13] T7 and SP6 RNA polymerase.

In claim 18 (amended). The method as defined in Claim 17, wherein said RNA polymerase activity is performed at temperature [ranged from about 35°C to about 85°C] about 37°C.

In claim 20 (amended). The method as defined in Claim 1, wherein said polynucleotide-tailed first-strand complementary DNAs are tailed by [terminal extension activity selected from the group consisting of terminal transferase and M-MulV reverse transcriptase with multiple copies of same kind of deoxynucleotides] terminal transferase activity.

In claim 30 (amended). The method as defined in Claim 22, wherein said RNA polymerase promoter is selected from the group consisting of [T3, T7, SP6 and M13] T7 and SP6 RNA polymerase promoter.

In claim 31 (amended). The method as defined in Claim 22, wherein said polynucleotide-tailed complementary DNAs are formed by [terminal extension activity selected from the group consisting of terminal transferase and M-MuLV reverse transcriptase] terminal transferase activity.

In claim 33 (amended). The method as defined in Claim 32, wherein said mixed polymerase activities are selected from the group consisting of [T3, T7, SP6, M13] T7 and SP6 RNA polymerases and Tth-like DNA polymerases with reverse transcriptase activity, C. therm. polymerase.

In claim 35 (amended). The method as defined in Claim 34, wherein said same deoxynucleotide is selected from the group consisting of [deoxyguanylate, deoxycytidylate, deoxyadenylate, deoxythymidylate and deoxyuridylate] deoxyguanylate, deoxycytidylate and deoxyadenylate.

REMARKS-General

1. The applicants respectfully submit that the list of patents, publications recited in the Description of The Prior Art of the original filed specification has also been listed in the Information Disclosure Statement filed with the specification of the instant invention. A copy of the Information Disclosure Statement filed is enclosed herewith for your reference.

Response to Objection of the Specification under 35USC132

2. The applicants respectfully submit that the text added to pages 6 and 15 in the Amendment A filed January 03, 2001 have been cancelled, so as to overcome the objection of the amendment A as introducing new matter into the disclosure.

Response to Rejection of Claims 1 to 36 under 35USC112

3. The applicants respectfully submit that the original claims 4-6, 19, 21, 24, 27, 28, and 36 are cancelled and the original claims 10-14, 16-18, 20, 30-31, 33, and 35 are amended to particularly point out and distinctly claim the subject matter of the instant invention, as pursuant to 35USC112.

4. Regarding to the rejection of claims 1 to 36 under 35USC112, first paragraph, as containing subject matter which was not described in the specification in such as way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

5. The applicants respectfully submits that detailed information of background skill and basic technology has been disclosed in the Examples 1 to 5 as well as the cited reference (Lin et. al. *Nucleic Acid Res.* 27:4585-4589 (1999) of the specification of the instant invention. In other words, all necessary information for person who skilled in this art has been sufficiently disclosed in the present application.

6. The amended claims 1-3, 7-18, and 20 are narrowed to match the stepwise description of the first preferred embodiment, referring to Fig. 1 and Example 5 in the specification of the instant invention. The amended claims 22-23, 25-26, and 29-35 are also narrowed to match the major steps of the description of the second and third preferred embodiments, referring to Figs. 2 and 3, and Examples 1-4.

7. Both the claims and specifications of the instant invention are amended above to ensure all the pending claims 1-3, 7-18, 20, 22-23, 25-26, and 29-35 are supported and described in the amended specification. Although a more detailed description of the preferred embodiments is provided in the Figures, Examples and amended specification, the practical simplicity of instant invention has been proven by the submitted results of Fig2. 4a-4b and a previously cited publication (Lin et. al. *Nucleic Acid Res.* 27: 4585-4589 (1999)) whose methodology is similar to the Example 5.

8. Accordingly, the "unpredictable factors" mentioned in the Office Action has been clarified by the previously provided evidences, proving that the feasibility of current invention is experimentally practical under described conditions in the specification. In fact, this technology is currently a routine performance for several laboratories in the Department of Pathlogy, Keck School of Medicine, University of Southern California. The development processes of the present invention did take years, but *the real practice of the current preferred embodiments can be completed within about four to five hours for a person skilled in simple molecular biology.*

9. It is because the instant invention is an in-vitro enzymatic procedure, the physiological and chemical conditions are usually provided by specially designed buffers rather than cellular conditions, which have been well described in the Examples 1-5 of the specification of the instant invention. These artificially buffered conditions ensure the success of an optimized enzymatic reaction, such as reverse transcription, terminal transferase tailing, polymerization and in-vitro transcription.

10. Similar kinds of buffered conditions are also required for all previously patented inventions involving in a variety of enzymatic reactions, such as U.S. Patent Numbers 4683202, 4965188, 5514545, 5795715, 5817465, 5888779, and many others. It is because the present invention is neither an in-vivo nor a chemical reaction, it is suggested that the chemistry and cellular physiology do not cause uncertainty in the previous and our enzymatic inventions under in-vitro buffer condition.

11. In view of above, the applicants respectfully believe that the amended claims 1-3, 7-18, 20, 22-23, 25-26, and 29-35 are all supported by the description of the originally filed specification of the invention in such a way as to reasonably convey to one skilled

in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention because of the following reasons.

12. Applicant believes that for all of the foregoing reasons, all of the claims 1-3, 7-18, 20, 22-23, 25-26, and 29-35 are in condition for allowance and such action is respectfully requested.

Respectfully submitted,

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